ELECTRON MICROSCOPIC EXAMINATION OF SUBCELLULAR FRACTIONS

I. The Preparation of Representative Samples from Suspensions of Particles

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ABSTRACT

A method is described for preparing, by filtration on Millipore filters, very thin (about 10 μ) pellicles of packed particles. These pellicles can be embedded in Epon for electron microscopic examination. They are also suitable for cytochemical assays. The method was used with various particulate fractions from rat liver. Its main advantages over the usual centrifugal packing techniques are that it produces heterogeneity solely in the direction perpendicular to the surface of the pellicle and that sections covering the whole depth of the pellicle can be photographed in a single field. It thus answers the essential criterion of random sampling and can be used for accurate quantitative evaluations.

The electron microscopic examination of subcellular fractions is usually performed on material collected by centrifugation. When the particles belong to various categories differing in sedimentation coefficient or in density, as is usually the case, their distribution in the pellet is far from homogeneous. The resulting heterogeneity does not occur solely in the direction of the centrifugal field, a difficulty which could be obviated by the preparation of pellets sufficiently thin to be examined over their whole depth, as was done by Ernster et al. (4). In our experience, this heterogeneity also extends along the surface of the pellet, causing particles of different types to occur with differing frequencies in the center and in the periphery of the preparation. For this reason, a truly quantitative evaluation of the morphological composition of a fraction packed by centrifugation requires scanning in at least two directions, a laborious procedure in view of the amount of material which has to be processed for convenient handling. Thus there is an obvious need for a method that would allow a rapid and accurate morphological evaluation of subcellular fractions. The problem is mainly one of adequate sampling.

Since any physical procedure used to pack heterogeneous particulate material is likely to introduce some heterogeneity, our attempts were aimed primarily at restricting this heterogeneity to a single direction of space and at minimizing the dimension of the sample in this direction. A very thin (about 10 μ) pellicle, eventually organized along its depth but not in any other direction, would meet these requirements. As already mentioned, we found centrifugation inadequate for the preparation of such pellicles, and we turned instead to filtration procedures which gave satisfactory results with various subcellular fractions.

MATERIALS AND METHODS

Subcellular Fractions

Female rats of a Wistar strain were used in all experiments. Fractions were isolated from normal liver according to the scheme of de Duve et al. (3). The large granule fraction is equivalent to the sum of

the M and L fractions, and the microsomal fraction to the P fraction of these authors. Except in the case of preparation of the microsomal fraction, animals were starved for 16 hr before sacrifice. The purified lysosomal fraction, obtained after injection of Triton WR-1339 (Rohm & Haas, Philadelphia, Pa.), was prepared by the method of Trouet (9).

Fixation

The material was fixed by mixing dilute particle suspensions in sucrose solution with 2–50 times their volume of an ice-cold solution containing 1.5% of glutaraldehyde in 0.05 M phosphate buffer, pH 7.4.

Filtration

In the first trials, the commercially available Filterfuge tube (International Equipment Co., Needham Heights, Mass.) was used as such. In this device, a Millipore filter (Millipore Filter Corp., Bedford, Mass.) with a diameter of 13 mm is fitted at mid-height in a stainless steel centrifuge tube assembly. Filtration in the centrifuge was very slow and it was found more convenient to accelerate filtration by gas pressure over the solution. For this purpose, the Filterfuge assembly was modified as shown in Fig. 1. The upper part of the tube was replaced by a plexiglas chamber *C* of the same inner diameter and 4 cm high, closed by a plug *A* held in place by a screw *B*. The total volume of chamber *C* is about 3 ml. A piece of tubing passing through the plug connects the chamber either with the outside or with a tank of compressed air, through a three-way valve whose positions are commanded electronically by the current flowing through two stainless steel conductivity probes sealed at mid-height in the walls of the chamber. The circuit is wired in such a way that the chamber is kept under pressure as long as fluid is present between the probes and is brought back to atmospheric pressure once the probes cease to be immersed in fluid. To avoid sudden pressure changes, a needle (No. 26 gauge) is inserted between the valve and the plexiglas chamber. An even pellet is obtained only if the filter is kept horizontal. A special tube holder built for this purpose consists of a metal block with a water level, leveling screws, and accurately machined holes into which the filtering units are held tightly in correct vertical position. During operation, the assembly is kept in a refrigerator.

A volume of the fixed suspension, varying between 0.3 and 1.2 ml depending on the type of preparation, and containing 150 μg of protein or less, is first pipetted above the filter of a precooled unit. Buffer of the same concentration as present in the fixative is then carefully layered on top of the sample with a syringe fitted with a needle bent at right angle. The difference in density between the buffer and the fixed

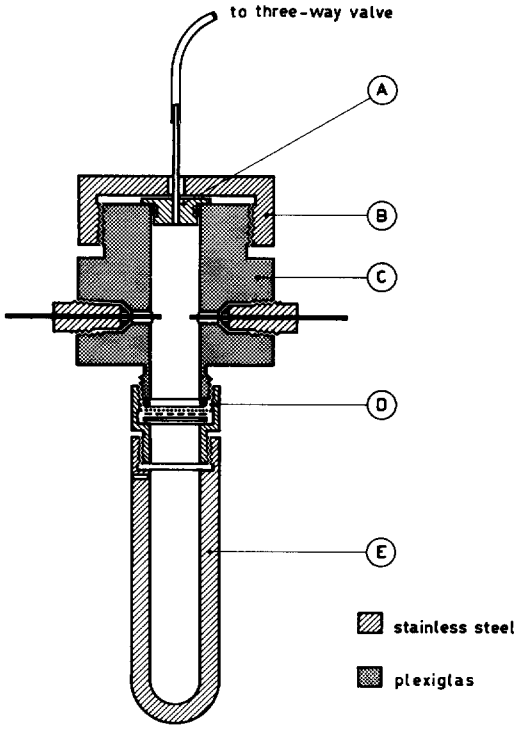

FIGURE 1 Filtration Unit. Part *D* and *E* are the standard parts of the Filterfuge Tube Kit (International Equipment Co, No. 1199). Part *D* includes a Teflon gasket, a supporting screen, a Millipore filter and a Teflon O-ring. Part *C* is the plexiglas chamber with the two conductivity probes. Part *A* is a plug held in place by the screw *B* and provided with a connection to the gas valve.

sample, which contains glutaraldehyde, prevents mixing of the two layers. Fluid is added first until the conductivity between the probes increases sharply; this provides a reference level. A volume of buffer equivalent to that of the sample plus 0.2 ml is then added and the unit is closed. Activation of the electric valve sets the liquid under pressure, keeping it as such until the fluid level drops below the conductivity probes, at which time the particles have been packed on the filter and washed with 0.2 ml of buffer. The pressure was adjusted to filter the samples in 20–50 min. A pressure of 3.6 kg cm^{-2} or less was usually sufficient. The pore size of the filters was selected according to the dimensions of the particles investigated: 0.01 μ for microsomes and 0.1 μ for mitochondrial and lysosomal fractions. In the latter case, some particles smaller than the pores, ribosomes, glycogen, etc., may have been lost through the filter, but they undoubtedly represented a negligible part of the material, since the procedures used to

prepare the particulate fractions eliminated most of the small particles. In experiments designed specifically for the study of the smaller components, 0.01 μ pores obviously should be preferred.

Postfixation, Dehydration, and Embedding

Preliminary trials showed that particles were lost from the upper part of the pellicle during postfixation and dehydration. It was found that the losses could be prevented by covering the sample with another Millipore filter coated with a thin layer of red blood cells. These protective discs were prepared by Buchner filtration of 10 ml of a 100-fold dilution of heparinized rabbit blood through a Millipore filter of 47 mm diameter and 1.2 μ pore size. Several 13 mm discs were punched out from this filter.

After filtration, the upper chamber *C* of the unit is unscrewed and the fluid remaining above the sample is removed by careful capillary suction with filter paper. Then the tube *E* and Teflon O-ring are removed, and the Millipore filter is raised on its supporting grid and covered with the protective disc in such a way that the layers of granules and of red blood cells are sandwiched between the two Millipore filters. A Teflon O-ring is put on top of the second filter and the two filters are then clamped between the stainless steel grid and the Teflon O-ring with a clamp of the screw compressor type which allows convenient handling. After a brief immersion in the buffer, the preparation is postfixed for 20 min in 1% of buffered osmium tetroxide, rinsed again in buffer and in 25, 50, 75 and 95% ethanol for about 3 min each. As a routine precaution, all the preceding steps are performed at 0°C, although it is possible that manipulations at room temperature would not harm the preparation. Dehydration is completed by three rinses in absolute ethanol at room temperature.

Then the clamp is unscrewed, and the Millipore sandwich is taken off with small tweezers and transferred to a low beaker containing propylene oxide. After 16 hr exposure to this solvent, the Millipore filters are completely dissolved, leaving a thin brittle pellicle formed by the apposed layers of particles and of red blood cells. By gently shaking the fluid, it is easy to insert below the pellicle a small aluminum dish, 2 cm wide and 4 mm deep, in which a 1 mm layer of Epon (5) has been prepolymerized. Careful raising of this dish makes it possible to remove the pellicle without handling. Then the propylene oxide present in the dish is sucked out and replaced first with a 50% mixture of Epon and propylene oxide, and then with pure Epon which is polymerized for 18 hr at 60°C. The embedded sample is detached by bending the aluminum dish.

Cytochemistry

When the cytochemical test for acid phosphatase was carried out, the sample was fixed with 1.5% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.4, and filtered as described above. The resulting pellicle was not coated with a protective disk, but was removed as such, rinsed for a few minutes in pure buffer, and immersed in an incubation mixture prepared according to Miller and Palade (6), but with sucrose omitted. Sometimes several fragments cut from a single pellicle were incubated under different conditions or used for optical controls. Preparations were incubated either at room temperature for 20 min or at 0°C for an hour. The pellicles were then rinsed for 5 min in 0.05 M cacodylate buffer, pH 7.4, and postfixed in the same buffer containing 1% of osmium tetroxide. Controls were run in an incubation mixture without β-glycerophosphate; postfixation was then performed in phosphate-buffered osmium tetroxide in order to detect any nonspecific binding of the lead.

TABLE I

Filtration Conditions

Fraction	Amount of protein in the samples	Pore size of filter	Pellicle thickness
	μg	μ	μ
M + L	100–150	0.1	6–12
Purified lysosomal	60	0.1	5
Microsomal	80	0.01	13

Sectioning

Sections were prepared with an LKB Ultrotome I microtome, equipped with a diamond knife. The specimen was trimmed to provide sections perpendicular to the surface of the pellicle; it was oriented to obtain an angle of 45° between the pellicle plane and the knife edge. This orientation favors smooth cutting, friction on the specimen being constant during the cutting stroke.

Some trouble was experienced in getting good sections. It appears that the Epon mixture had to be considerably softer with our material than with tissue blocks. This is because the section is made largely through pure Epon. The correct hardness could be found easily by polymerizing a set of different mixtures of Epon, trying them on the microtome, and selecting the one with the best cutting qualities. With our products, the best results were obtained when the pellicles were embedded in a medium containing 70% of the A and 30% of the

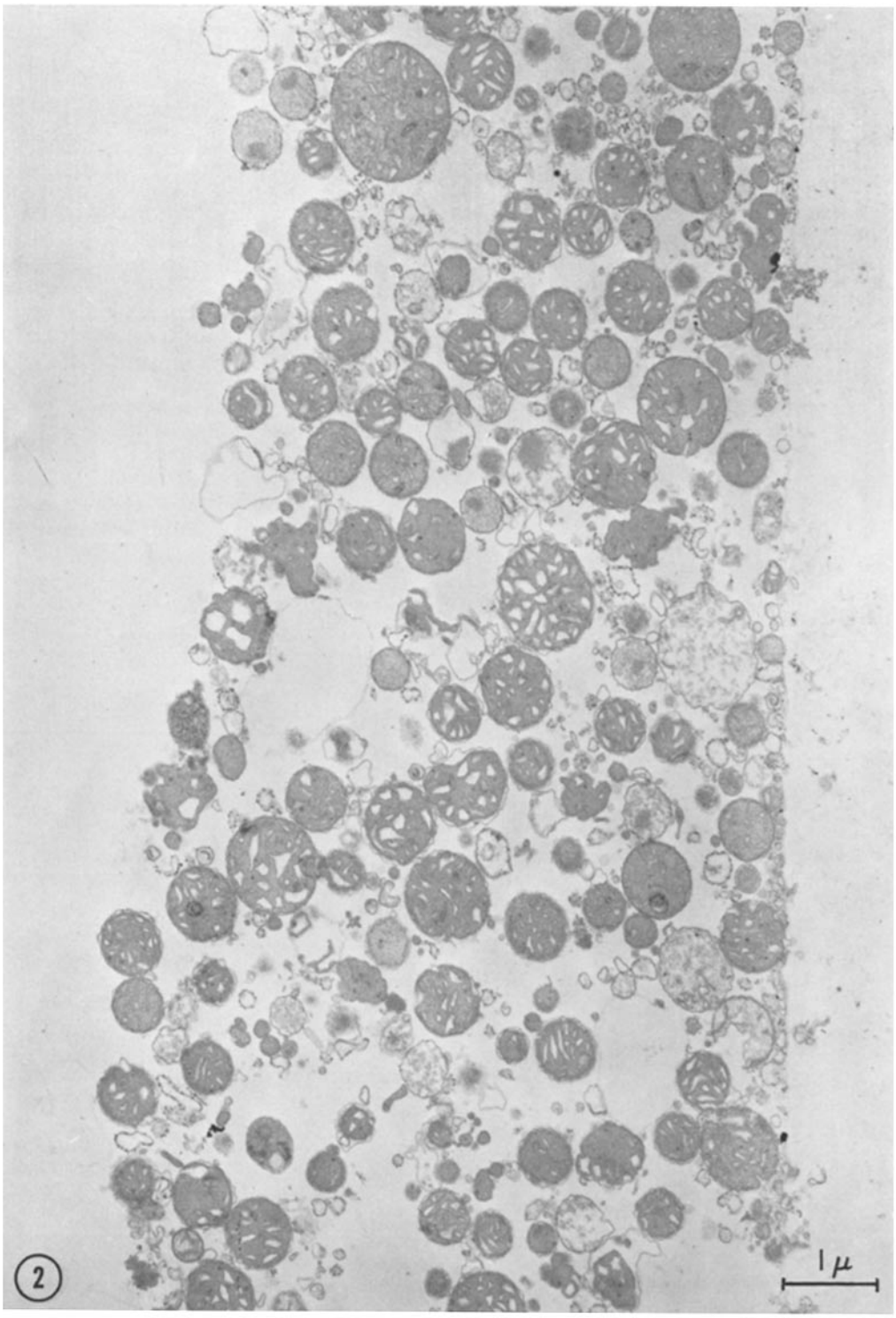

FIGURE 2 Pellicle of an M + L fraction from rat liver cut perpendicular to its surface. The lower surface of the pellicle, which was in contact with the Millipore filter, is on the right side in this micrograph; it has a sharp boundary in contrast to the more irregular limit of the upper surface. × 14,500.

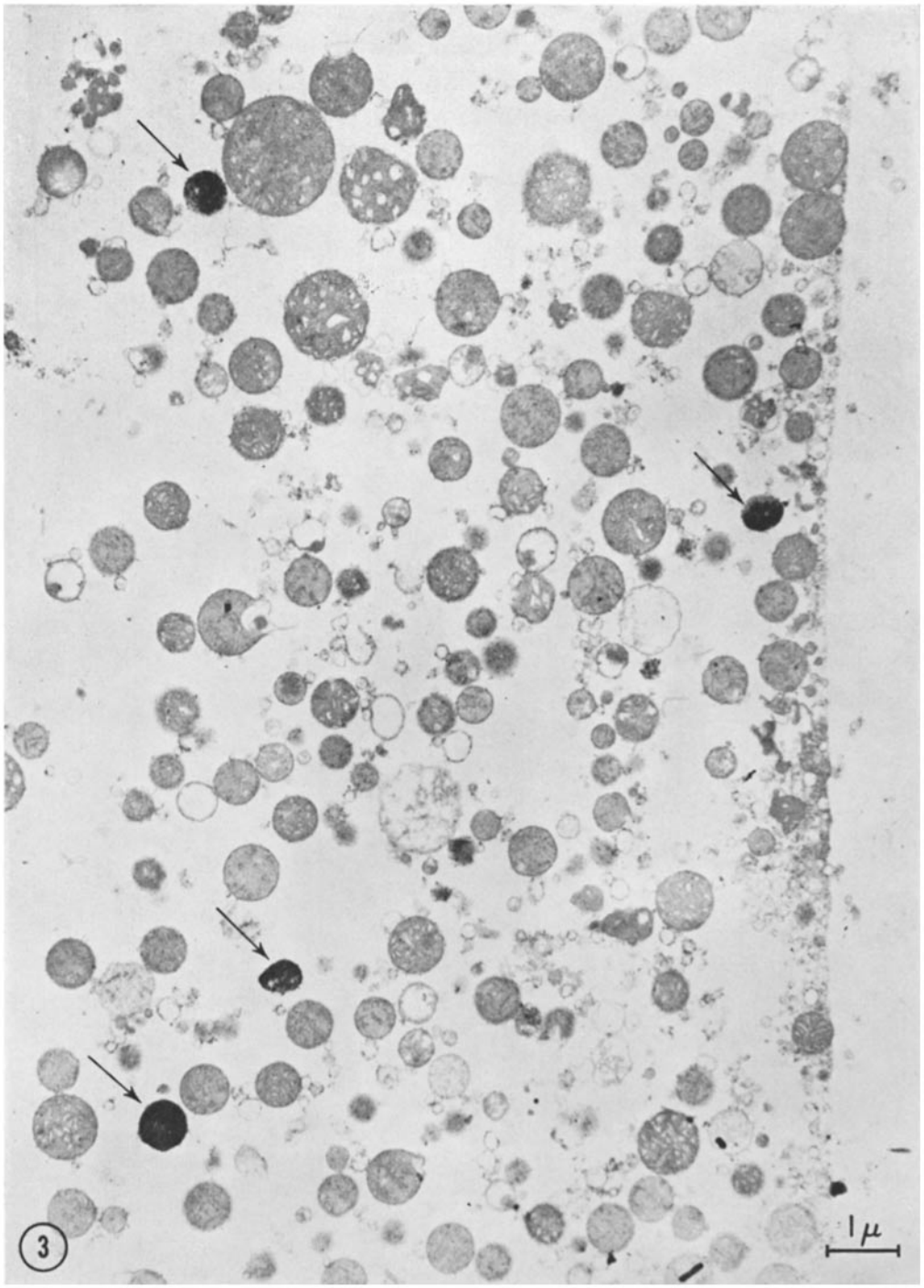

FIGURE 3 Cytochemical test for acid phosphatase on a M + L fraction. Four granules show positive reaction (arrows). Note loose packing as compared to the preparation of Fig. 2. The protective layer of red blood cells was omitted here. × 11,000.

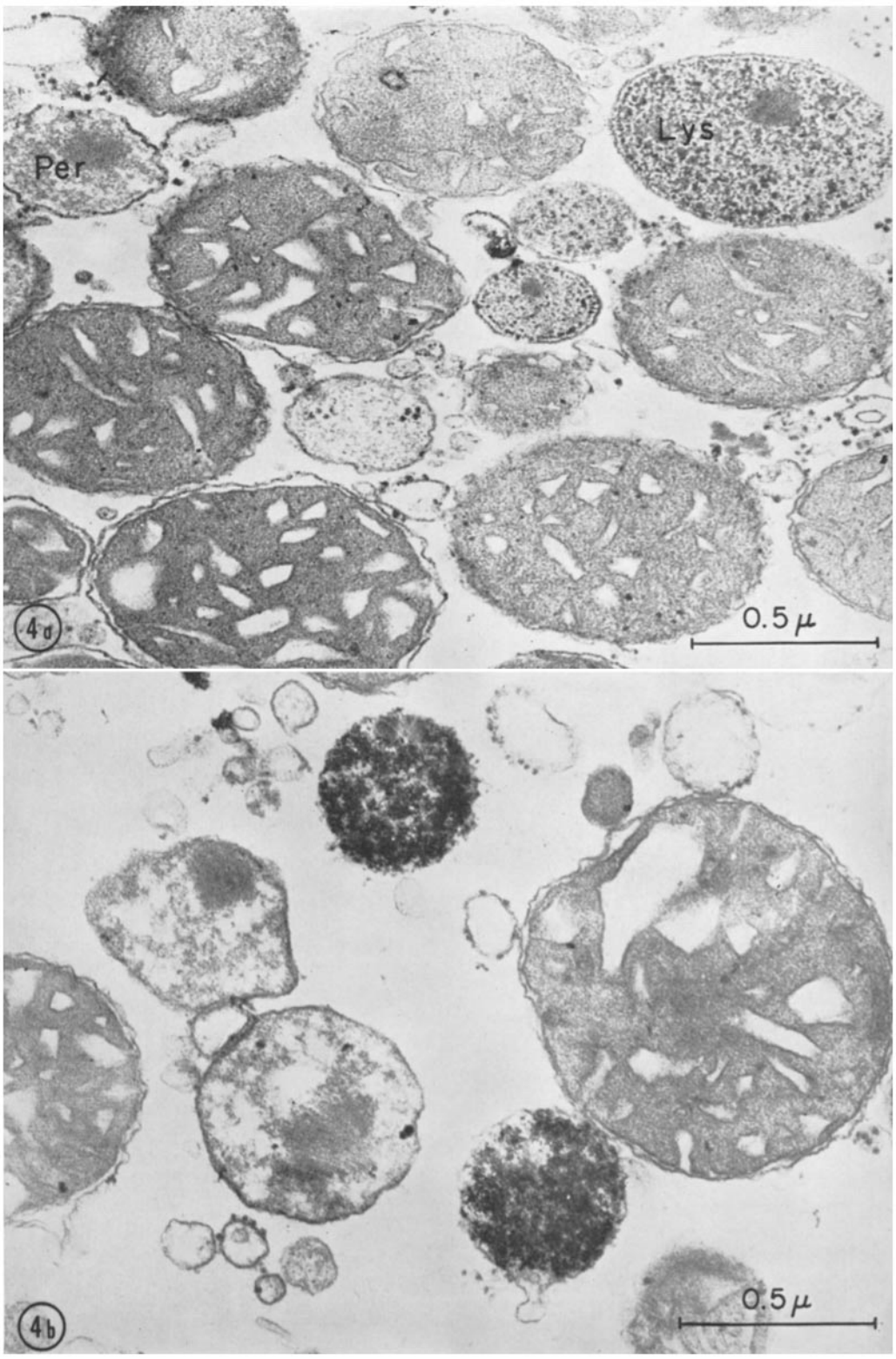

FIGURE 4 *a* Preparation similar to that in Fig. 2, at higher magnification (*Lys* = lysosome; *Per* = peroxisome). × 58,000.

FIGURE 4 *b* Preparation similar to Fig. 3 at higher magnification. Two granules show an acid phosphatase reaction. Mitochondria and peroxisomes are negative. × 60,000.

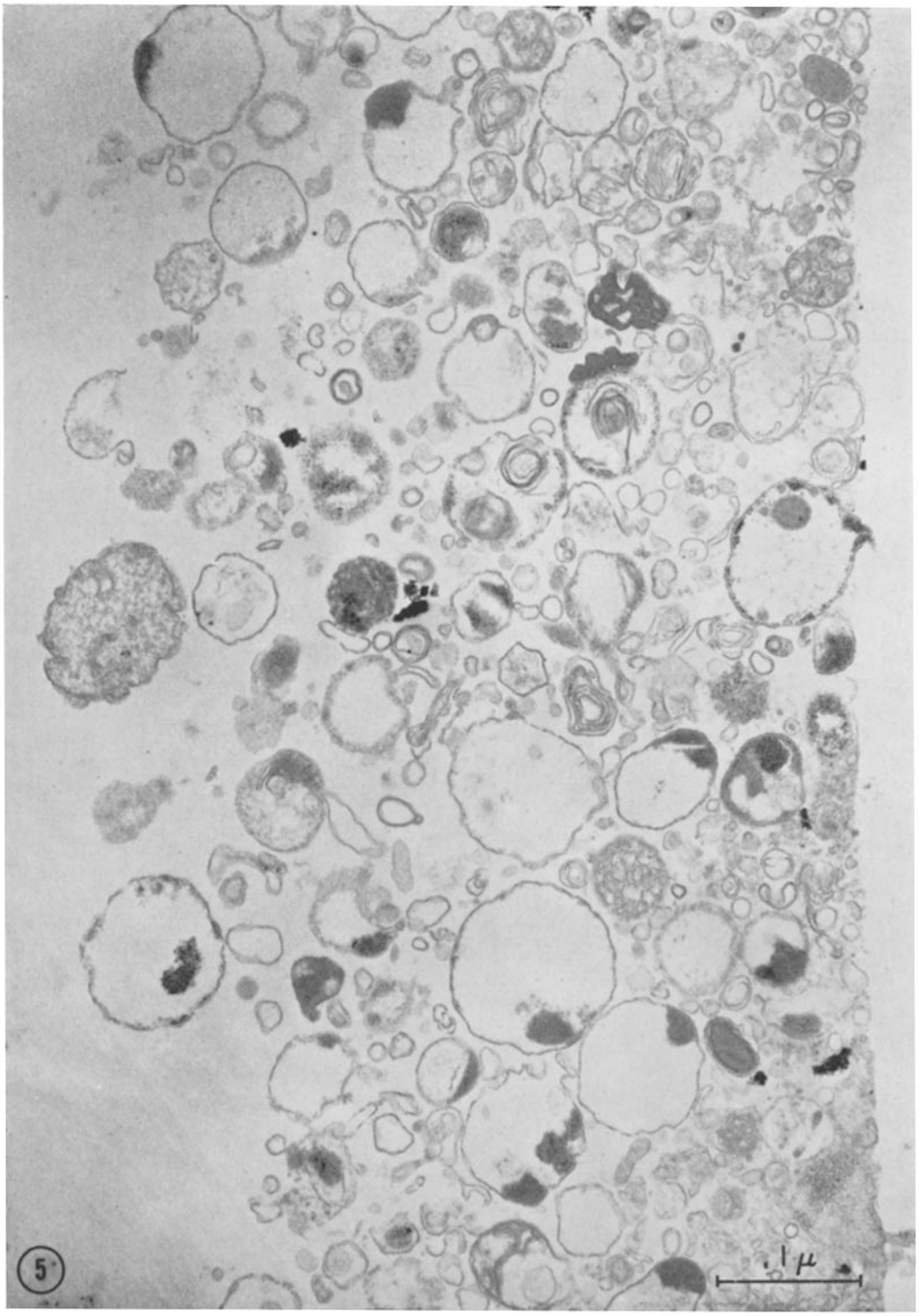

FIGURE 5 Purified lysosomal fraction, obtained after injection of Triton WR-1339. Small elements are accumulated in the lower part of the pellicle. × 21,000.

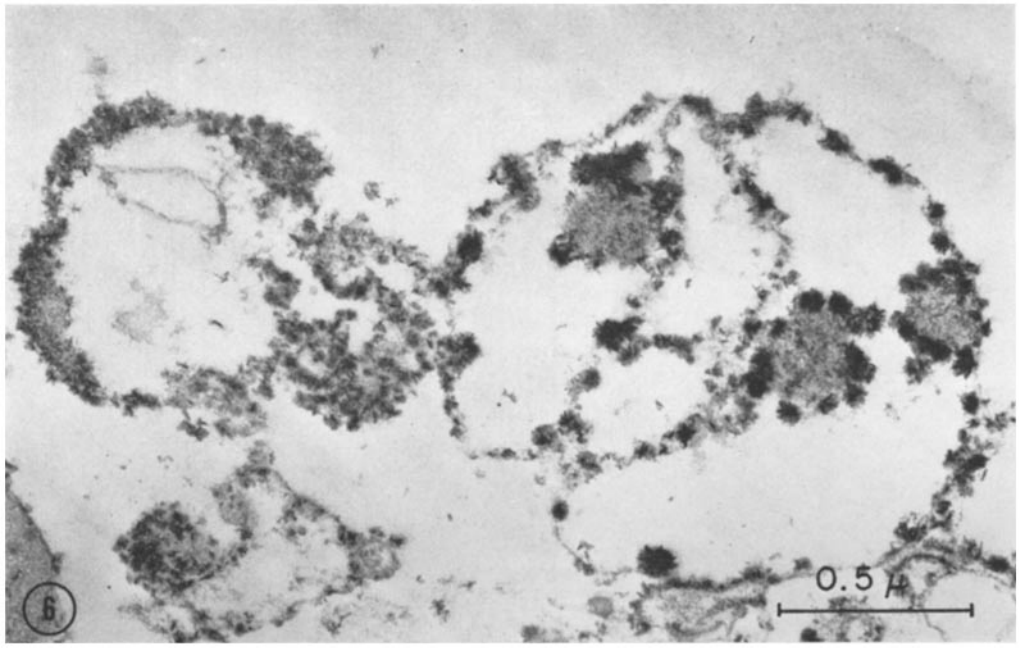

FIGURE 6 Cytochemical test for acid phosphatase on the same preparation as in Fig. 5. × 57,000.

B mixtures of Luft (5) (while the reverse ratio is routinely used to embed rat liver blocks).

The sections were stained with 1% uranyl acetate in 50% ethanol for 10 min, and then with lead citrate (7) for another 10 min. They were examined at 60 kv, with a Siemens Elmiskop I microscope.

RESULTS

General Appearance of the Preparations

The filtration conditions which provided satisfactory preparations are given in Table I. Pellicles were easily photographed over their whole depth, which varied between 5 and 13 μ. Their appearance is illustrated by the electron micrographs shown in Figs. 2 to 7.

Generally, the red blood cell layer was not in close contact with the granules; sometimes, the two layers were as far apart as 20 μ. However, the intervening space was practically empty and the very few granules present in it could be neglected. The red blood cell layer seemed to stabilize the pellicle primarily by protecting it against erosion by liquid flows during its various transfers. At the lower part of the pellicle, the particles were tightly packed; the sharpness of the boundary shows that no granules were lost from this side. The morphological preservation of the granules of various types seemed as good as it did when they were collected by centrifugation. The material was organized in the direction perpendicular to the pellicle surface. It appears that small elements crept between the bigger ones and accumulated in the lower part of the preparation. However this type of heterogeneity is unimportant if the pellicle is examined over its whole depth.

The cytochemical test for acid phosphatase could be applied to the pellicles without difficulty (Figs. 3 *b*, 4, and 6). Controls, without β-glycerophosphate, were negative.

The general appearance of the M + L fractions illustrates the heterogeneity of preparations isolated by differential centrifugation; although mitochondria are the main component, contaminants are varied and numerous. The microsomal fractions gave pellicles in which small elements were closely packed in the lower part of the preparation. Attempts were made to use smaller amounts of material, but the pellicle became mechanically to weak and did not withstand the embedding manipulations. The pictures obtained with purified lysosomal fractions isolated after injection of Triton WR-1339 were similar to the ones published by Wattiaux et al. (10), but they

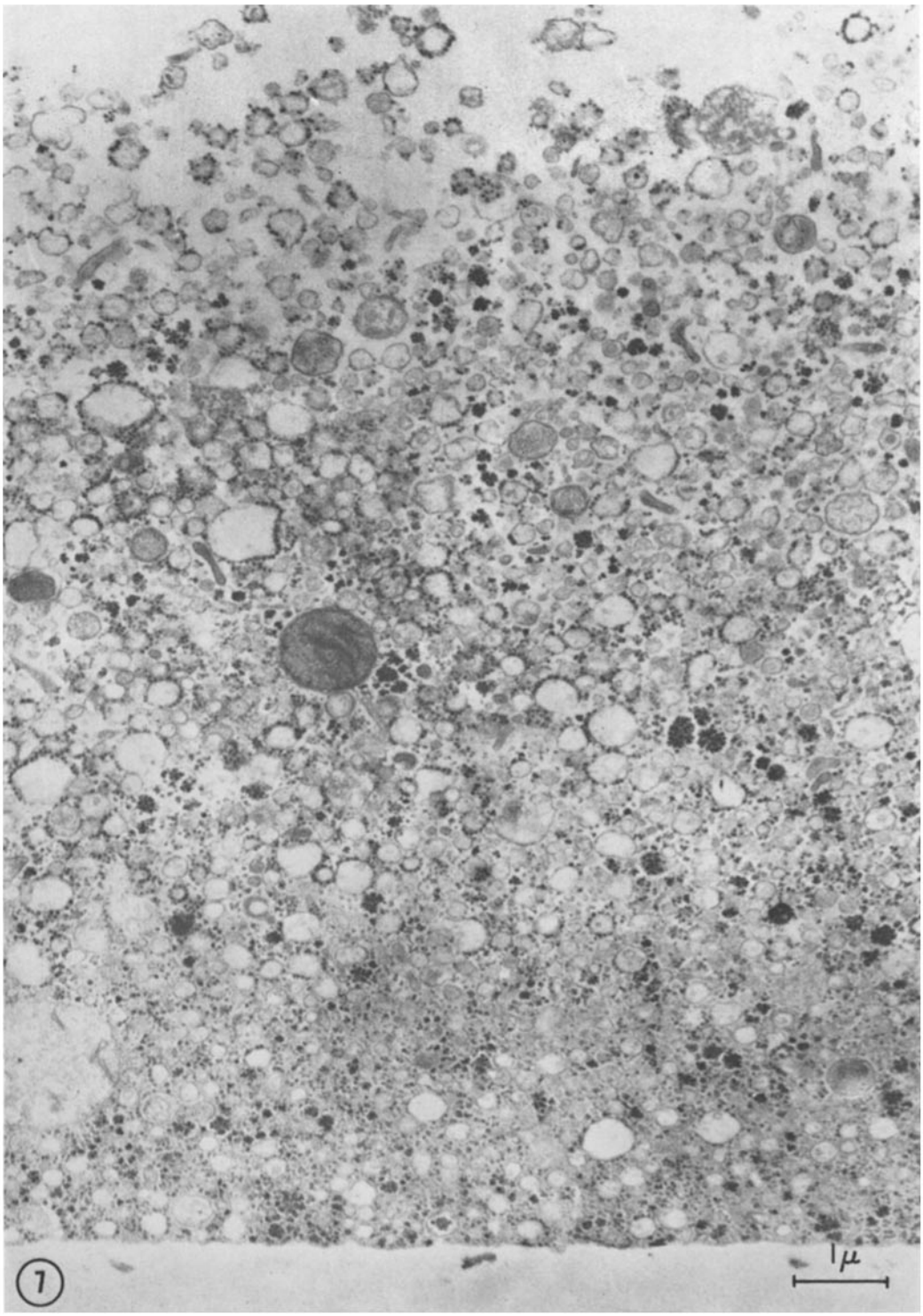

FIGURE 7 Microsomal fraction. Note close packing, especially in the lower part of the pellicle. × 14,000.

showed more clearly the presence of numerous small vesicles, in addition to the large Triton-filled lysosomes which were, as expected, the most conspicuous component. The small vesicles could be small lysosomes, pinocytotic vacuoles, or parts of the smooth endoplasmic reticulum. In the latter case, they must be formed of a special type of membrane material essentially devoid of glucose-6-phosphatase, since the preparations examined contained less than 0.05% of the hepatic content in this enzyme. Some of the small vesicles were positive for acid phosphatase but more weakly so than the large lysosomes, which showed heavy lead deposits near the membrane and in the vicinity of the dense regions which probably represent remnants of their normal structure.

Homogeneity of the Preparations

In no case could any obvious evidence of heterogeneity be detected between different sections made through the sample pellicle. To verify this point more accurately, a pellicle prepared from an M + L fraction was sectioned at four different sites extending from the periphery to the center, separated by at least 1 mm. Three distinct micrographs of each section were analyzed over a width of about 18 μ. The average pellicle thickness was 11.6 μ with a standard deviation of 0.8 μ; the area examined in each micrograph was thus about 200 μ^2. The number of recognizable profiles of mitochondria, microbodies or peroxisomes (2), and lysosomes was counted (Table II). If the pellicle is homogeneous over its entire surface, the number of profiles per micrograph should follow a Poisson distribution; therefore, the variance should be equal to the mean. As shown by the results in Table II, the observed variances are slightly greater than the corresponding means. However, the F test (8) performed with n equal to 11 for the variance and to infinity for the mean indicates that these differences are not significant statistically ($P > 0.2$, in all three cases). These measurements thus failed to reveal any significant heterogeneity of the pellicle.

TABLE II

Homogeneity of a Pellicle Prepared by Filtration

Parameter	Mitochondria	Peroxisomes	Lysosomes
Average number of profiles per micrograph	145	9.2	5.6
Variance	194	9.5	6.6

DISCUSSION

The filtration technique described in this paper shows considerable advantages over the usual methods of centrifugal collection. It is at least equally fast and easy to perform and provides the electron microscopist with a specimen, each section of which may be considered truly representative of the whole preparation. Thus the essential requirement for random sampling is satisfied. The technique is applicable to minute quantities of material and, therefore, can be used in conjunction with analytical fractionation methods where high resolution generally is obtained at the expense of yield (1). Amounts of material even smaller than those reported here could be processed by decreasing the filter diameter by a factor of two or even four. The method is flexible and is likely to be applicable to particles of both larger and smaller size than the ones investigated in this work, since filters with pores ranging from several μ to 0.01 μ are commercially available. Pellicles prepared as described also lend themselves easily to cytochemical demonstrations of enzymic activities and may even provide very suitable systems for the study of the kinetics of cytochemical reactions. Finally, they are very well suited for quantitative work. It must be noticed, in the latter respect, that the number of particle profiles seen in a section does not supply a direct estimate of the number of particles in the preparation, since the probability of cutting a particle also depends on its size. The counting methods and the mathematical procedures required for obtaining quantitative measurements of particle size and number will be presented in a subsequent paper.

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